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α1,2-Fucosyllactose Does Not Improve Intestinal Function or Prevent *Escherichia coli* F18 Diarrhea in Newborn Pigs

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ABSTRACT

Objectives: Infectious diarrhea, a leading cause of morbidity and deaths, is less prevalent in breastfed infants compared with infants fed infant formula. The dominant human milk oligosaccharide (HMO), α-1,2-fucosyllactose (2′-FL), has structural homology to bacterial adhesion sites in the intestine and may in part explain the protective effects of human milk. We hypothesized that 2′-FL prevents diarrhea via competitive inhibition of pathogen adhesion in a pig model for sensitive newborn infants.

Methods: Intestinal cell studies were coupled with studies on cesarean-delivered newborn pigs (n = 24) without (control) or with inoculation of enterotoxigenic *Escherichia coli* F18 (7.5×10^{10}/day for 8 days) fed either no (F18) or 10 g/L 2′-FL (2FL-F18).

Results: In vitro studies revealed decreased pathogen adhesion to intestinal epithelial cells with 2′-FL (5 g/L; $P < 0.001$). F18 pigs showed more diarrhea than control pigs ($P < 0.01$). Administration of 2′-FL to F18 pigs failed to prevent diarrhea, although the relative weight loss tended to be reduced (−19 vs −124 g/kg, $P = 0.12$), higher villi were observed in the distal small intestine ($P < 0.05$), and a trend toward increased proportion of mucosa and activities of some brush border enzymes in the proximal small intestine. In situ abundance of α-1,2-fucose and *E coli* was similar between groups, whereas sequencing showed higher abundance of Enterobacteriaceae in F18, *Enterococcus* in control and Lachnospiraceae in 2FL-F18 pigs.

Conclusions: 2′-FL inhibited in vitro adhesion of *E coli* F18 to epithelial cells, but had limited effects on diarrhea and mucosal health in newborn pigs challenged with *E coli* F18.

Key Words: α-1,2-fucosyllactose, *E coli*, human milk oligosaccharide, infection, newborn pigs

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What Is Known

- Breastfeeding reduces the risk of infectious diarrhea.
- Human milk oligosaccharides prevent bacteria-induced diarrhea in older infants but limited information is available from newborns.
- Piglets are sensitive to enteric infections, making them good models to investigate dietary effects on the gut in sensitive newborn infants.

What Is New

- α-1,2-Fucosyllactose reduced *Escherichia coli* F18 epithelial adhesion, but failed to prevent diarrhea in newborn F18-challenged pigs. Marginal improvements were seen for intestinal structure and function.
- α-1,2-Fucosyllactose supplementation has limited protective effects on the newly colonized, immunocompromised newborn intestine. Benefits of human milk oligosaccharide may be highly age, diet, and dose dependent.

Compared with formula feeding, breastfeeding reduces the risk of infectious diarrhea, a worldwide problem and a leading cause of infant deaths, especially in the third world (1). Both nutritional and bioactive components in human milk may be responsible for this protective effect, and the human milk oligosaccharides (HMOs) have been suggested to play a role. They pass largely undigested through the infant intestine (2), but are the third most abundant nutrient group in human milk (3,4), whereas they are present in only trace amounts in cow's milk (5). One proposed mechanism of HMOs is its decoy effect whereby HMOs competitively prevent bacterial adhesion to the intestinal epithelium due to

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structural homology to mucosal glycoconjugates that represent bacterial adhesion sites (6). In line with this, antimicrobial effects of HMOs have been demonstrated in several in vitro studies (7–9).

The most abundant HMO in human milk is α-1,2-fucosyllactose (2′-FL) (5). Cell and animal studies have shown that 2′-FL is structurally homologous to fucosylated intestinal adhesions sites (H-2 antigens) for different bacteria, including pathogenic bacteria such as *Campylobacter jejuni* and enterotoxigenic *Escherichia coli* (ETEC) strains (9,10). The decoy effect of 2′-FL has been demonstrated in mice and human intestinal biopsies, in which a dose of 10 g/L competitively inhibited adhesion of *C jejuni* (9). Administration of HMOs has also been shown to reduce intestinal lesions in a newborn rat model of necrotizing enterocolitis (11), and infants receiving mother's milk with high concentrations of 2′-FL had fewer cases of diarrhea caused by *Campylobacter*, *E coli* toxins or caliciviruses (12). Fewer episodes of both respiratory and enteric infections were also observed among infants who had a high intake of HMOs (13).

In pig production, ETEC strains such as *E coli* F18 are a major problem causing diarrhea in weanling pigs. The virulence of *E coli* F18 depends on bacterial fimbriae binding to the intestinal F18 receptor, H-2, a blood group antigen that carries a α-1,2-fucosylated group (10). *E coli* F18 virulence further depends on the host expression of *FUT-1*, the gene responsible for fucosylation of the H-2 antigen (14–16). Previously, *FUT-1* expression, and thereby the sensitivity toward *E coli* F18 infection, was not thought to occur in pigs before 3 weeks of age (16,17). We, however, recently documented similar expression levels of *FUT-1* in preterm and term newborn pigs and in weanling pigs, and we demonstrated that *E coli* F18 induced diarrhea in newborn pigs deprived of sow's milk (18). Based on this, we hypothesized that dietary 2′-FL inhibits *E coli* F18 adhesion to intestinal epithelial cells and prevents F18 infectious diarrhea in newborn cesarean-delivered pigs. Immaturity of the intestine and immune system makes this animal model very sensitive to maldigestion and enteric pathogens and hence also to the diet factors that may reduce pathogen influence and increase mucosal immunity.

Antiadhesive effects of 2′-FL in vitro was tested by incubation of porcine jejunal epithelial PSIc1 cells, with *E coli* F18. The 2′-FL tolerance and optimal doses of *E coli* F18 inoculation were tested in 2 studies with newborn pigs, followed by an intervention study with daily inoculations of 7.5×10^{10} *E coli* F18 and administration of 10 g/L 2′-FL mixed into a cow's milk–based infant formula. Prevalence of diarrhea was the primary endpoint. Secondary endpoints were body weight gain, blood gas values, intestinal weight and proportions, mucosal enzyme activity, and permeability. Finally we quantified in situ abundance of *E coli* and the local endogenous intestinal production of α-1,2-fucose.

MATERIALS AND METHODS

In Vitro Cell Adhesion

Culture of E coli F18 and E coli ATCC 25922

The challenge strain (9910297–2^{STM}, O138:$F18^{14}$) and an F18-negative control strain (American Type Culture Collection, *E coli* ATCC 25922) were first grown overnight at 37°C on blood agar plates. A loop full (10 μL) of colony material was then suspended in 4 mL sterile phosphate-buffered saline (PBS) and poured onto an isosensitest agar (Oxoid, Roskilde, Denmark) supplemented with Alizarin yellow 0.06% w/v (Merck) chosen for optimal expression of F18 fimbria (19). Excess suspension was discarded before overnight incubation at 37°C and 10% CO_2. *E coli* was harvested from the plates in PBS and diluted in antibiotics-free Advanced Dulbecco's modified Eagle's medium (DMEM) for further analyses to OD_{600} 0.5 (Pharmacia Biotech GeneQuant pro, Cambridge, UK) corresponding to 3×10^8 cfu/mL.

Inhibition of E coli F18 Adhesion to PSIc1 Cells

PSIc1 cells (Bionutritech, Lunel, France) originating from jejunal epithelium of an adult pig were cultured in DMEM supplemented with 2% fetal bovine serum (FBS), 2 mmol/L GlutaMAX, 40 U/mL penicillin, and 40 μg/mL streptomycin (all from Gibco, Life Technologies, Carlsbad, CA) at 37°C and 5% CO_2 in a humidified atmosphere. At 90% to 95% confluence, cells were harvested by trypsinization using ×10 Trypsin:EDTA (Gibco, Life Technologies) and cultured in T75 culture flasks and 12-well plates (TPP, Trasadingen, Switzerland) for genotyping and adhesion assay, respectively. TaqMan single nucleotide polymorphism genotyping was performed as previously described (18) to investigate guanine (G)/adenine (A) polymorphism at nucleotide 307 of the *FUT-1* gene, in which $M307^{G/G}$ or $M307^{G/A}$ represents susceptibility to *E coli* F18 adhesion (15).

First, it was tested if *E coli* F18 and the control *E coli* ATCC 25922 strain adhered to PSIc1 cells. After cultivation in 12-well plates, each well of confluent PSIc1 cells was added 500 μL *E coli* solutions of OD_{600} 0.5 to OD_{600} 0.0005, giving a cell:bacteria ratio of 1:1000 to 1:1. Cells and *E coli* were then coincubated at 37°C and 5% CO_2 in a humidified atmosphere in medium without antibiotics. After 2 to 3 hours, each well was washed twice in cold PBS, and cells were harvested in 1 mL PBS, vortexed thoroughly and plated in ×10 dilutions on LB agar plates for bacterial enumeration. To visualize adhesion of *E coli* F18, 3 hours coincubation and subsequent washing in PBS was performed as above on 8 well CultureSlides (BD Falcon, VWR, Herlev, Denmark) in a cell:bacteria ratio of 1:100. Bacteria were stained with LIVE/DEAD *Bac*Light stain (Molecular Probes, Life Technologies) according to the manufacturer's protocol and visualized using a florescence microscope (Carl Zeiss, Oberkochen, Germany).

To test the antiadhesive effects of 2′-FL, the PSIc1 cells and *E coli* F18 or *E coli* ATCC 25922 (both at OD_{600} 0.5) were incubated as above, but before incubation the medium was supplied with 2′-FL (produced utilizing technology from Glycosyn LLC, Medford, MA, by DuPont Nutrition and Biosciences ApS, Copenhagen, Denmark) in concentrations of 1 or 5 g/L compared with a control only added saline. Lactose and maltose were used as control sugars at equivalent molar concentrations. Bacteria were enumerated on agar plates as above. The level of inhibition was calculated based on 8 to 14 replicates for *E coli* F18 and 4 replicates for *E coli* ATCC 25922.

Pig Studies

All animal procedures were approved by the Danish National Committee on Animal Experimentation.

Experiment 1: 2′-FL Tolerance Study

A blinded study was set up to test pig tolerability toward 2′-FL to verify that large doses of 2′-FL would not induce osmotic diarrhea. Two sows (Danish Landrace × Large White × Duroc) were selected for homozygosity ($M307^{G/G}$) of the *FUT-1* gene to ensure at minimum heterozygosity of the offspring (18). Thirty pigs were delivered at term by cesarean section and transferred to individual temperature controlled incubators and, still under anesthesia, fitted with a vascular catheter in the umbilical aorta and an orogastric catheter. During the first 24 hours, pigs were infused with plasma from the sow (4–7 mL/kg) to ensure passive

immunization and with parenteral nutrition (7 mL/kg/h of Kabiven and Vamin, Fresenius Kabi, Bad Homburg, Germany) adjusted to the nutrient requirements of pigs. The above procedures has been thoroughly described previously (20). To standardize the initial gut colonization within each litter, each pig received an oral fecal suspension from the sow on day 1 (1 mL with totally 2×10^6 cfu). A baseline blood sample was taken from the umbilical catheter and analyzed for blood gasses on a GEM©Premier3000 (Instrumentation Laboratory, Zaventem, Bruxelles, Belgium). From day 2, full enteral feeding was given as boluses of 15 mL/kg every 2 hours with the exception of a 4 hours break during night, reaching a daily dose of 150 mL/kg/day. Pigs were fed a milk replacer (MILEX, Arla Foods, Viby, Denmark) with added protein (50 g/L Lacprodan DI-9224, Arla Foods) as previously described (18). Based on birth weight and sex, the pigs were allocated into 4 diet groups, supplementing the milk replacer with 2′-FL to a total concentration of 10 g/L (FL-10, n = 7), 5 g/L (FL-5, n = 8), 2 g/L (FL-2, n = 7), or no 2′-FL (controls, n = 8).

Body weight and temperature was recorded every morning before feeding, and the consistency and amount of feces was scored morning and evening, where 0 was absence of feces, 1 was normal feces, 2 was pasty feces, 3 was droplets of watery feces, 4 was moderate amounts of watery feces, and 5 was extensive amounts of watery feces. Also, the level of hydration, as assessed by skinfold test and piglet physical activity, was assessed twice daily. In case of severe dehydration and apathy, the pigs were euthanized before the scheduled euthanasia and tissue collection on day 5. Intestinal permeability was determined as the post-mortem urine ratio of lactulose and mannitol after oral administration of the 2 sugars (0.5 and 0.3 g/kg, respectively) 3 hours before euthanasia (21). At euthanasia, blood gasses were measured as above, and 10 cm sections of proximal and distal small intestine were sampled for determination of the wet and dry proportion of mucosa (21).

Experiment 2: E coli F18 Dose-Response Study

The optimal *E coli* F18 inoculation dose was then identified in a dose-response study. Thirty-one term pigs were delivered by caesarean section from 2 sows, fitted with orogastric and umbilical catheters, immunized with sow's serum, and nourished by parenteral nutrition as described for experiment 1. For the following 4 days, the pigs were fed 15 mL/kg/3 hours (Milex formula as above supplemented with 23 g/L Lacprodan, DI9224) until euthanasia on day 5. The pigs were randomly assigned to 4 different treatment groups, controls (CON, n = 8) or 3 doses of F18, low dose (LOW, 1×10^7 CFU/day, n = 9), medium dose (MED, 2×10^8 CFU/day, n = 7), and high dose (HIGH, 8×10^9 CFU/day, n = 7). *E coli* F18 was cultured overnight as described above, and harvested with 10 mL PBS. From the first day and throughout the experiment all LOW, MED, and HIGH pigs were inoculated daily with 1 mL F18 suspension. The suspension was given through the orogastric catheter followed 5 mL of the Milex formula. Initial gut colonization was standardized by giving 1 mL of maternal fecal suspension, as described in experiment 1. Body weight was recorded daily and fecal consistency and amount was scored twice daily as described for experiment 1. Likewise, clinical condition, hydration, intestinal permeability, and mucosal proportions were measured. Sections of distal small intestine were formalin fixed and paraffin embedded for fluorescence in situ hybridization (FISH) with an *E coli*–specific 16 s rRNA probe (S-S-*E coli*-1161, 5′ GCATAAGCGTCGCTGCCG 3′[23]) labeled with isothiocyanate derivative (CY3, red signal) to evaluate abundance of *E coli* in the proximal and distal small intestine. In short, 3 μm tissue sections were mounted on glass slides (Superfrost/Plus slides, Menzel-Gläser, Braunschweig, Germany), deparaffinated in xylene, dehydrated in ethanol, hybridized overnight with the probe at 49°C using Shandon Coverplates and Sequenza immunostaining workstations (Thermo Scientific, Waltham, MA) followed by 3× washing in hybridization buffer (Tris 0.1 mol/L, NaCl 0.9 mol/L, 0.1% SDS, pH 7.2) and 3× washing in washing buffer (Tris 0.1 mol/L, NaCl 0.9 mol/L, pH 7.2). An Axioimager M1 epifluorescence microscope (Carl Zeiss) was used for visualization and images were obtained using an AxioCam MRm version 3 monochrome camera and multidimensional acquisition in the software AxioVision version 4.5 (Carl Zeiss). Based on the abundance of *E coli*, each pig was given a standardized score where 1 = no bacteria, 2 = few bacteria, 3 = some bacteria, and 4 = many bacteria in the tissue section.

Experiment 3: 2′-FL F18 Infection Study

Finally, an intervention study was performed to test whether 2′-FL inhibited *E coli* F18 infectious diarrhea. Twenty-five pigs were delivered at term by cesarean section from 2 *FUT-1* homozygous sows (Danish Landrace × Large White × Duroc). As described above, the pigs were prepared with umbilical and orogastric tubes, infused with parenteral nutrition and sow's plasma, inoculated with maternal feces, and from day 2 transferred to full enteral feeding with boluses of milk replacer. Based on birth weight and sex, the pigs were allocated into 3 groups. One group was inoculated daily with 7.5×10^{10} *E coli* F18 (F18, n = 9), 1 group was given the same dose of *E coli* F18 plus 10 g/L 2′-FL in the milk replacer as described in the tolerance study above (2FL-F18, n = 8) and 1 control group received only milk replacer (control, n = 8). *E coli* F18 was cultured overnight at 10% CO_2 on Iso-sensitest agar with Alizarin yellow as described above, and harvested with 10 mL PBS, of which each pig received 1 mL. On day 1, *E coli* F18 was given 1 mL of the respective diets, while inoculations the following days were followed by enteral feeding. The sensitivity toward diets and pathogen inoculation were followed until euthanasia on day 8, unless severe dehydration and apathy required euthanasia beforehand.

Body weight was recorded every second day and clinical evaluation was assessed as above. Fecal consistency scores were evaluated morning and evening using a scoring system, where 0 = absence of feces, 1 = normal feces, 2 = pasty feces, and 3 = watery feces. Blood gasses at birth and at euthanasia, intestinal permeability, and mucosal proportions were obtained as above. Sections of proximal and distal small intestine were snap frozen for activity determination of 6 brush border enzymes (22).

Sections of distal small intestine were formalin fixed and paraffin embedded for villus height measurements (using the ImageJ software, version 1.22 c US National Institutes of Health, Bethesda, MD) and evaluation of mucosal damage and villus atrophy or degradation. The same tissues were evaluated for in situ *E coli* abundance evaluated by FISH as described for experiment 2. For microbiota analyses, total DNA of the colon content was extracted and the microbiota composition determined using tag-encoded 16S rRNA gene MiSeq-based high-throughput sequencing (Illumina, San Diego, CA), as previously described (23). The Quantitative Insight Into Microbial Ecology (QIIME, version 1.7.0) and UPARSE methods were used to analyze the sequencing data.

To determine the level of 2-linked fucosylation in the distal small intestine, the tissue sections were hybridized with α-1,2-fucose–specific lectin, Ulex europaeus agglutinin I (24) labeled with fluorescein isothiocyanate (green). In short, tissue sections were deparaffinated and dehydrated as above, washed in 1× PBS for 1 hour, permeabilized in 1× PBS with 1% TritonX for 1 hour, hybridized with 0.5% lectin in 1× PBS and 2% BSA protected from

light for 1 hour and finally washed in 1× PBS. All procedures were performed at room temperature. Sections were evaluated under microscope as above and given a score where 0 = no staining, 1 = few positive stained goblet cells, 2 = several positive stained goblet cells, mainly in the crypt area *or* moderate luminal staining, 3 = abundant positive stained goblet cells along the entire mucosal lining, and 4 = abundant positive stained goblet cells along the entire mucosal lining together with extensive luminal staining.

Statistical Analyses

Data analyses were performed in R version 3.0.1. Treatment effects on diarrhea and weight development were analyzed using the lmer function of repeated measurement analysis with litter and pig as random variable and the factors treatment and time as fixed variables. In vitro adhesion data and treatment effects on relative weight gain, intestinal parameters, blood parameters, FISH, and lectin hybridization from the pig studies were tested using the lmer function of linear mixed model analysis with sow as random variable and treatment, birth weight and sex as fixed variables. In case of significance, pairwise analysis using the glht function was employed to compare effects of the different treatments. Normal distribution of all measured parameters was tested by plotting the model residuals and by the qq-plot using the qqnorm function. All values given in the report are adjusted means and standard error of mean. Data are considered significant for $P < 0.05$.

RESULTS

In Vitro Adhesion

Genotyping of PSIc1 cells showed homozygosity ($M307^{G/G}$) for the F18 susceptible genotype of FUT1 (data not shown). Both *E coli* F18 and ATCC 25922 adhered to the PSIc1 cells, which was confirmed on culture slides by fluorescence microscopy for *E coli* F18 (Fig. 1A). High doses of 2′-FL (5 g/L) effectively inhibited *E coli* F18 adhesion ($P < 0.001$; Fig. 1B), whereas inhibition of *E coli* F18 adhesion was less effective but significant when lactose and to a lesser extend maltose (not shown) was added ($P < 0.001$ and 0.05, respectively). Adhesion of *E coli ATCC 25922* was not affected by 2′-FL or any of the control sugars (Fig. 1B).

Experiment 1: 2′-FL Tolerance Study

One control pig and 1 FL-5 pig were euthanized before completion of the protocol due to respiratory problems or apparent sepsis. Fecal scores from these 2 pigs were included in the final calculations. Although 3 FL-10 pigs (43%), 1 FL-5 (13%) and 2 FL-2 pigs (29%) had lost weight at euthanasia relative to their birth weight, there was generally a slight increase in weight during the experiment, with no difference between groups at any time point ($P = 0.3–0.7$; Supplemental Fig. 1A, *http://links.lww.com/MPG/A704*). Fecal consistency was noted from day 2 where feces first appeared and thus resulted in 6 observations in total per pig. Pigs from all treatment groups developed diarrhea (score 3–5) at some point; 3 controls (38%), 3 FL-2 pigs (43%), 4 FL-5 (50%) and 2 FL-10 pigs (29%), and mean fecal scores generally increased during the 5-day period with no difference between groups ($P = 0.3–0.8$; Supplemental Fig. 1B, *http://links.lww.com/MPG/A704*). Intestinal permeability, expressed as the lactulose/mannitol ratio, did not differ among groups (0.02–0.12 across groups). There were no group differences in dry weight mucosal proportions in the proximal small intestine or distal small intestine ($75 \pm 2\%$ and $73 \pm 2\%$, respectively).

The rectal temperature increased from $36.2 \pm 0.0^{\circ}C$ after delivery to $39.1 \pm 0.1^{\circ}C$ on the following day in which it remained stable until euthanasia. There was no difference between treatment groups and all pigs remained within the normal range during the experiment. Blood gas values showed no differences among groups, except that FL-2 pigs had lower hematocrit values than FL-10 pigs ($22.6 \pm 1.0\%$ vs $26.5 \pm 0.9\%$, $P < 0.05$). Across all groups, mean hematocrit decreased from $35.2 \pm 0.6\%$ at birth to $25.1 \pm 0.8\%$ at euthanasia ($P < 0.05$). At birth, mean p_{O_2} and p_{CO_2} was higher than p_{O_2} at euthanasia (119.5 ± 17.3 vs 52.3 ± 5.6 mmHg and 52.5 ± 1.5 vs 47.6 ± 1.8 mmHg, respectively, $P < 0.05$), whereas there was no overall difference in pH (pH 7.47 ± 0.01 vs 7.41 ± 0.03 at birth and euthanasia, respectively, $P = 0.13$). Blood glucose concentration increased from birth to euthanasia (2.6 ± 0.2 vs 5.2 ± 0.4 mmol/L, respectively, $P < 0.05$).

Experiment 2: E coli F18 Dose-Response

The incidence of diarrhea increased with time after inoculation ($P < 0.01$), and was dose dependent with more diarrhea in HIGH versus CON ($P < 0.05$), and a tendency to more diarrhea in MED versus CON and HIGH versus LOW pigs (both $P < 0.07$; Fig. 2). Body weight gain was lower in the HIGH group compared with CON ($P < 0.01$), but hydration score (1.06 ± 0.01), mucosal proportion ($77.8 \pm 3.9\%$), and intestinal permeability (0.017 ± 0.004) did not differ among groups. The abundance of *E coli* in the proximal (1.1 ± 0.04) and distal small intestine (1.8 ± 0.15) as measured by FISH analyses did not differ between groups.

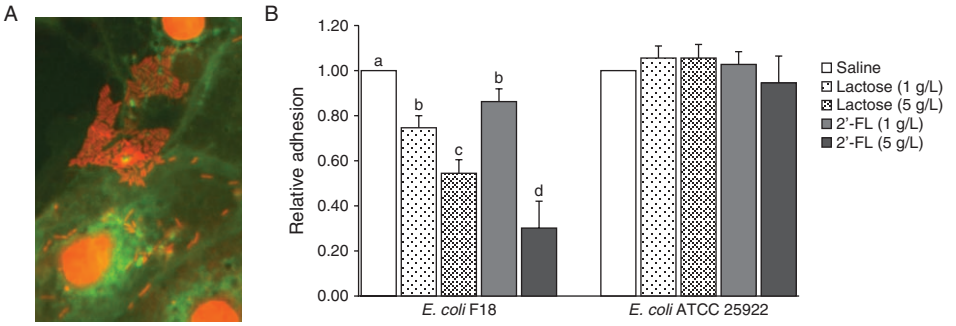

FIGURE 1. Microscopic visualization of *Escherichia coli* F18 adhesion to PSI cells (A) where bacteria are stained with a red fluorescence dye. Mean relative adhesion of *E coli* F18 and control *E coli* ATCC 25922 to PSI cells in vitro after addition of 2′-FL and lactose (1 and 5 g/L, B). Different superscript letters indicate significant differences ($P < 0.05$).

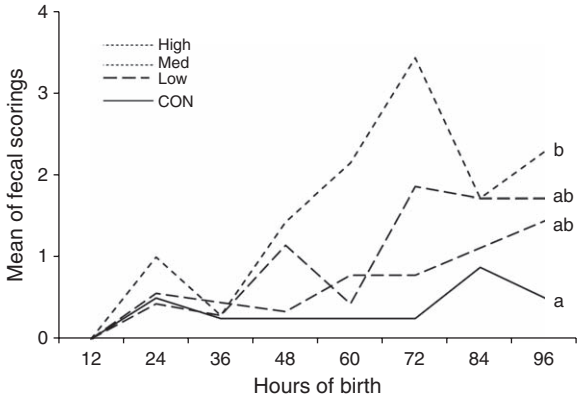

FIGURE 2. Mean of fecal scorings in pigs receiving either no *Escherichia coli* F18 (CON) or increasing doses of *E coli* F18 (LOW, MED, or HIGH) during the 5-day *E coli* F18 dose-response study (experiment 2). Different subscript letters indicate overall significant differences.

Experiment 3: 2′-FL-F18 Pig Infection Study

Because there was no difference in tolerability between the different 2′-FL doses in experiment 1, a dose of 10 g/L 2′-FL was used in the *E coli* F18 infection study to increase the *E coli* F18 antiadhesive potential of the intervention. Also the high *E coli* F18 dose was chosen to ensure consistent diarrhea development (experiment 2). All 9 F18-pigs, 6 out of 8 2FL-F18 pigs and 2 of 8 control pigs were euthanized before completion of the protocol because of extensive diarrhea and poor clinical condition (Fig. 3A). Nevertheless, there was no difference in the mean age at euthanasia among the treatment groups (154 ± 9, 141 ± 9, and 132 ± 8 hours for control, 2FL-F18, and F18 pigs, respectively, $P = 0.20$). At euthanasia, control and 2FL-F18 pigs tended to show less relative weight loss than F18 pigs ($P = 0.08$; Fig. 3B). All pigs developed diarrhea during the experiment, but overall both F18 and 2FL-F18 pigs had more severe diarrhea, and earlier onset of diarrhea, than controls (mean score 2.0 for F18 and 2FL-F18 pigs vs 1.2 in control pigs, $P < 0.01$; Supplemental Fig. 2, *http://links.lww.com/MPG/A705*).

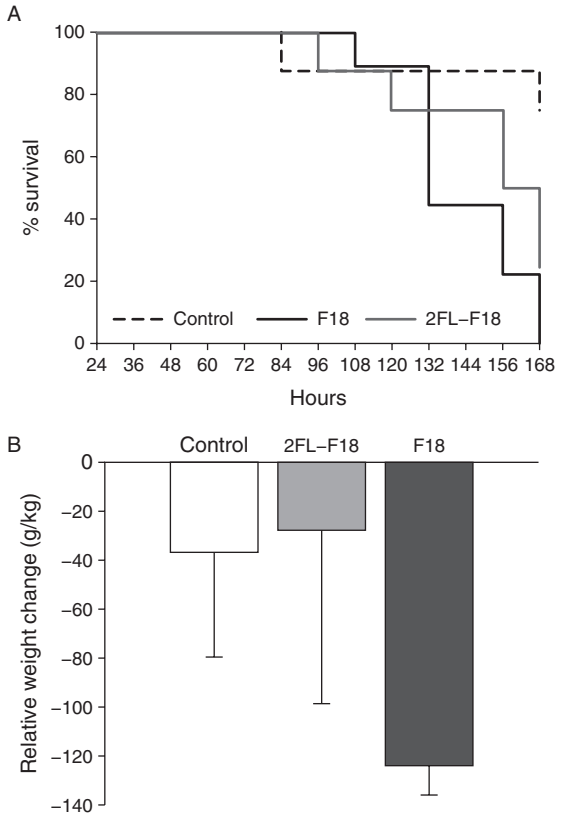

FIGURE 3. Survival curves for the 3 treatment groups (A) and relative weight change (B) during the 8-day 2′FL-F18 infection study (experiment 3). Values in B are means and standard error of mean. There were no differences between groups.

The rectal temperature increased from $36.0 \pm 0.0^{\circ}C$ after delivery to $38.5 \pm 0.1^{\circ}C$ the following day, where it remained stable until euthanasia. There was no difference between treatment groups and all pigs remained within the normal range with no evidence of fever (sepsis). Because of poor clinical status at euthanasia, blood gasses were not obtained from 6 F18 and 6 2FL-F18 pigs and 2 controls. Plasma glucose concentrations were elevated in F18 pigs, compared with 2FL-F18 and controls (13.5 ± 1.4 vs 6.7 ± 1.3 and 5.1 ± 1.3 mmol/L, respectively, $P < 0.01$). Also, hematocrit was increased in F18 pigs, compared with 2FL-F18 pigs and controls ($41.0 \pm 2.8\%$ vs $23.2 \pm 2.2\%$ and $28.5 \pm 1.2\%$, respectively, $P < 0.01$). Blood pH was lowest for F18 pigs (7.17 ± 0.11 vs 7.40 ± 0.09 and 7.27 ± 0.06 for F18, 2FL-F18, and controls, respectively), but the differences were not significant ($P = 0.34$ and 0.73, respectively).

As a result of severe dehydration, urine was only obtained from a few pigs, and lactulose and mannitol were therefore not analyzed for permeability measures. By histological evaluation, mucosal damage in terms of destructed villi with total or partly sloughing of enterocytes was observed in 25%, 14%, and 44% of controls, 2FL-F18, and F18 pigs, respectively ($P = 0.32$). F18 pigs had shorter villi than 2FL-F18 pigs and tended to have shorter villi than controls ($P < 0.05$ and $P = 0.06$, respectively, Table 1). Dry weight mucosal proportions in the proximal and distal small intestine was not different between groups ($P = 0.47$ and 0.41, respectively, Table 1).

The activities of 6 brush border enzymes in the proximal and distal small intestine were not different among groups (Table 1); however, the 2FL-F18 pigs showed the highest mean values for all 3 disaccharidases and aminopeptidase N in the proximal intestine, relative to F18 pigs, but the differences did not reach significance (Table 1, $P = 0.07–0.37$). Values for aminopeptidase A and N and dipeptidyl peptidase IV were higher in the distal small intestine than in proximal intestine ($P < 0.05$ for all 3 enzymes). FISH analyses revealed low total abundance of *E coli* in the distal small intestine (Fig. 4A), where 36% of the tissues were negative for *E coli*. There were no differences in mean *E coli* abundance among the treatment groups (Fig. 4B), and no difference in numbers of tissues negative for *E coli* (22%, 57%, and 38% for F18, 2FL-F18, and controls, respectively, $P = 0.3–0.8$) or numbers of tissues with high abundance (score 3–4; 29%, 22%, and 50% for F18, 2FL-F18, and controls, respectively). Furthermore there was no significant correlation between FISH score and mean fecal scores ($P = 0.26$). The F18 pigs had higher relative abundance of an unclassified Enterobacteriaceae genus, compared with control pigs (red bar, $P < 0.01$; Fig. 4C), whereas *Enterococcus* tended to be highest for control pigs (dark blue bar, $P = 0.058$). Compared with 2FL-F18 pigs, the control pigs had higher *Enterococcus* levels ($P < 0.05$). The 2FL-F18 pigs had an increased relative abundance of an unclassified Lachnospiraceae genus, compared with F18 pigs (purple bar, $P < 0.05$).

TABLE 1. Villus height, mucosal proportion, and activity of intestinal brush border enzymes in the proximal (Prox) and distal (Dist) small intestine of control, 2FL-F18, and F18 pigs

	Control		2FL-F18		F18	
	Prox	Dist	Prox	Dist	Prox	Dist
Villus, μm		358.3 ± 18.8		351.3 ± 14.1		303.5 ± 14.4*
Mucosa, %	50.0 ± 5.9	60.4 ± 1.6	57.3 ± 5.1	60.0 ± 1.3	49.1 ± 4.4	56.3 ± 1.4
Sucrase, U/g	0.34 ± 0.16	0.25 ± 0.04	0.58 ± 0.17	0.24 ± 0.04	0.26 ± 0.16	0.27 ± 0.04
Maltase, U/g	2.01 ± 0.60	2.02 ± 0.19	3.90 ± 0.65	1.99 ± 0.21	2.25 ± 0.57	1.87 ± 0.17
Lactase, U/g	5.15 ± 1.77	3.48 ± 0.68	8.54 ± 1.86	3.47 ± 0.74	5.18 ± 1.65	4.26 ± 0.63
ApN, U/g	2.23 ± 0.58	3.53 ± 0.62	3.00 ± 0.60	2.62 ± 0.66	1.96 ± 0.57	3.41 ± 0.61
ApA, U/g	0.88 ± 0.30	1.79 ± 0.28	1.31 ± 0.31	1.47 ± 0.29	1.16 ± 0.29	1.33 ± 0.27
DPPIV, U/g	0.73 ± 0.13	2.23 ± 0.58	1.07 ± 0.14	2.37 ± 0.51	0.91 ± 0.12	2.70 ± 0.44

Values are Mean ± standard error of mean.
*Villus height in F18 is significantly different from 2FL-F18 ($P < 0.05$). ApA = aminopeptidase A; ApN = aminopeptidase N; DPPIV = dipeptidyl peptidase IV.

Lectin hybridization revealed modest to high levels of α-1,2-fucose in the distal intestine (Fig. 4D), with strong staining of goblet cells, indicating endogenous production of α-1,2-fucose. A few pigs from all 3 treatment groups only displayed positive staining in the lumen and there were only few observations of α-1,2-fucose associated with the cell surface. The average score was not affected by treatment (2.0 ± 0.7, 2.5 ± 0.7, and 2.3 ± 0.7 for controls, 2FL-F18, and F18, respectively). There were no significant correlation between the amount of α-1,2-fucose and fecal scores but a negative correlation was observed between α-1,2-fucose score and *E coli* FISH score ($P < 0.01$, $r = -0.33$, data not shown).

DISCUSSION

Breastfeeding is strongly associated with a reduced risk of infectious diarrhea, especially in developing countries (1), and the effect may be due to numerous bioactive compounds in human milk. A compound that may have significant effect is 2′-FL, the major HMO in human milk, which has antiadhesive effects on the intestinal epithelium due to its structural homology with intestinal adhesions sites for pathogenic bacteria (9,10). This has been demonstrated for *C jejuni* at a dose of 10 g/L in mice and also for human intestinal biopsies (9). Absence of 2′-FL may be a significant factor for the increased risk of diarrhea when infants are fed cow's milk–based formulas and 2′-FL may help to prevent pathogenic adhesion to the intestinal mucosa. In line with our hypothesis, we confirmed epithelial antiadhesive effects in vitro of 2′-FL on the porcine pathogen, *E coli* F18. In our in vivo model of highly sensitive cesarean-delivered, formula-fed newborn pigs, high doses of 2′-FL were well tolerated but regardless, 2′-FL failed to prevent diarrhea associated with *E coli* F18 inoculation. Some clinical and intestinal estimates tended to be improved in the 2FL-F18 compared with F18 group (weight loss, blood hematocrit and pH, mucosal damage, villus height, and proximal intestine enzyme activities) but generally the differences did not reach statistical significance. Dietary 2′-FL may have more important effects in response to other types of enteric infections and later into postnatal life, but the effects on *E coli* F18 infection in a newly colonized, immature intestine just after birth are minimal.

First, we determined the *FUT-1* gene of the PSIc1 cells to be homozygous for *E coli* F18 sensitivity ($M307^{G/G}$) and demonstrated efficient *E coli* F18 adhesion, which was subsequently decreased 60% when 2′-FL at a dose of 5 g/L was added to the cell adhesion model. 2′-FL thereby showed more efficient inhibition than lactose, which is a common carbohydrate source in breast milk and many infant formulas, and 2′-FL was even more effective than maltose, also found in some infant formulas. Because 2′-FL specifically inhibited adhesion of *E coli* F18 and not of a control *E coli*, our in vitro data indicate that the antiadhesive effect of 2′-FL occurred via an F18 fimbria-dependent mechanism. Importantly, the effect was dose dependent, with no effect of 2′-FL at the low 1.0 g/L concentration. The 2′-FL concentrations applied in vitro relates to the physiological concentrations found in human milk, but may not be directly translated to the in situ situation, in which dilution by gastric and intestinal secretions and the mucus layer would reduce the level of 2′-FL in contact with the epithelium, depending on the infants health status. Preterm infants with very immature gastrointestinal functions and low digestibility may have reduced capacity to secrete these fluids and in situ concentrations may come closer to the raw milk concentrations. Considering the 5.0 g/L as a maximum of physiological relevant concentration, and the lacking effects at 1.0 g/L, the in vitro studies may confirm the minor effects of 2′-FL in the pig studies, even though the concentrations represents physiological levels. The dose-dependent effect implies that optimization of the dosage for in vivo administration is crucial to exert protection from *E coli* F18 infections. Therefore, we first tested 2′-FL in a dose-response newborn pig study comparing a low (1 g/L), medium (5 g/L), and high (10 g/L) dose of 2′-FL without *E coli* F18 infection. Considering a potential risk of osmotic diarrhea, the high dose was taken from the upper range of physiological concentrations in human milk (up to 8 g/L) (25–27). Even using a relatively high dose of 2′-FL, diarrhea was not prevented in newborn pigs inoculated with *E coli* F18. The explanation for the lack of effect may be related to the very immature state of the intestine and its colonization but also the dosing of 2′-FL and *E coli* F18. The 10 g/L 2′-FL used for the formula may not result in a comparable high concentrations at the epithelial surface since dilutions due to both endogenous mucus production and other epithelial secretions occur. In our in vitro studies on cells devoid of these physiological characteristics a dose of minimum 5 g/L was needed for significant antiadhesive effects and ex vivo studies in mice and human intestinal biopsies required a dose of 10 g/L to inhibit adhesion of *C jejuni* (9). The observed diarrhea in the 2FL-F18 group may be partly an osmotic diarrhea because HMOs are generally indigestible (2) leading to some accumulation of HMO in the colon where they become fermented by bacteria. Excessive fermentation increases the osmolality of the luminal contents and thereby induce osmotic diarrhea (28). It is therefore possible that the diarrhea observed in the 2FL-F18 and F18 pigs was of a different type, being more

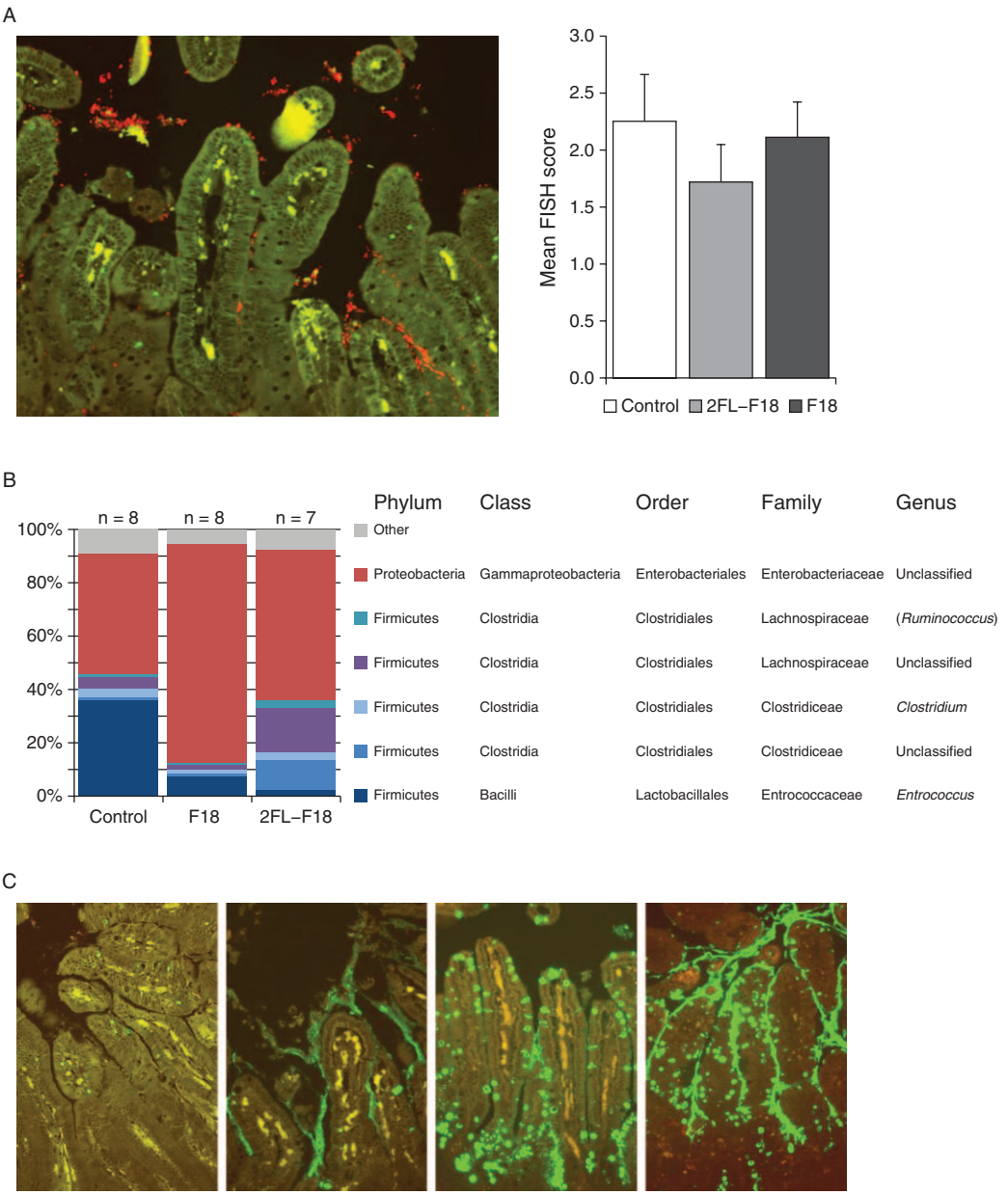

FIGURE 4. Representative tissue from the distal small intestine showing high abundance of *Escherichia coli* (red signal) visualized by FISH (A), and abundance of *E coli* in the treatment groups in the 2′FL F18 infection study (experiment 3) (B, means and standard error of mean). Average relative abundance distribution of the major genera in colon contents of control, F18, and 2FL-F18 pigs by 16S rRNA gene MiSeq-based high-throughputs sequencing (C). Tissue sections from the distal small intestine hybridized with fluorescence- (fluorescein isothiocyanate [FITC]) labeled lectin (ulex europaeus agglutinin I [UEA-I]) specific for α-1,2-fucose (D). The sections shown indicate tissues with fucosylation scores 1 (most left), 2, 3, and 4 (most right). FISH = fluorescence in situ hybridization.

osmotic for the 2FL-F18 pigs and more infectious for the F18 group, in the latter group leading to more mucosal disruption as shown for infectious diarrhea induced by ETECs (29). On the contrary, the tendencies for 10 g/L 2′-FL to reduce the weight loss, blood parameters, and mucosal lesions in F18-infected, formula-fed piglets may result from direct host tissue responses via immune modulation, or modulation of epithelial glycosylation processes, as shown for 2′-FL and other HMOs (6,24).

We based the *E coli* F18 dose of 7.5×10^{10} cfu/day on findings from experiment 2 in which a high dose (8×10^{9} cfu/day) more effectively induced diarrhea than lower doses. A previous study indicated an apparent upper tolerability limit of 2.6×10^{11} cfu/day (unpublished results). In the present 2′-FL intervention study, diarrhea was more severe in both inoculated groups compared with control pigs. FISH analysis, however, did not reveal higher intestinal abundance of total *E coli* in the inoculated groups of experiments 2 and 3. Still, data from this and an earlier study (18) document a robust and consistent pathogen-specific induction of diarrhea by *E coli* F18 in newborn, cesarean-delivered pigs. Replacement of formula with bovine colostrum did not prevent *E coli* F18 infection in

this model (unpublished observations), despite the higher contents of oligosaccharides in colostrum versus mature milk. Both sow's and cow's milk contain significantly lower amounts of fucosylated oligosaccharides than human milk (27), and the contents of 2′-FL in sow's milk has not yet been reported. Regardless, it appears that the intestine of suckling pigs is not likely to be highly dependent on soluble 2′-FL from milk for natural pathogen protection. Conversely, our in situ hybridization analyses with the α-1,2-fucose–specific lectin, ulex europaeus agglutinin I, revealed high endogenous secretion of α-1,2-fucose in the pig small intestine. The physiological effect of these may possibly override the modest additional effect of 2′-FL supplemented via the formula diet. In contrast to humans, pigs may therefore rely more on endogenous α-1,2-fucose, explaining the lacking or modest effects of 2′-FL supplementation on enteric F18 infection in this study. Although the endogenous α-1,2-fucose could not prevent *E coli* F18 diarrhea in our newborn pig model, there was a negative correlation between the level of endogenous α-1,2-fucose and the abundance of *E coli* in general and this may reflect some effect of α-1,2-fucose on intestinal colonization.

Our in vitro studies showed that 2′-FL at a concentration of 5 g/L inhibited *E coli* F18 adhesion to porcine intestinal epithelial cells. The lacking correlation between the *E coli* FISH score and diarrhea score may suggest that even low titers of *E coli* F18 can induce diarrhea. The binding of *E coli* F18 to luminal H-2 epitopes may also not be the only mechanism whereby the pathogens adhere to the epithelium and become virulent. In weanling pigs, *E coli* F18 diarrhea was most prevalent in susceptible pigs being homo- or heterozygote for the functional *FUT-1* gene, but also unsusceptible pigs developed diarrhea (14). A similar finding was reported ex vivo (16) and indicates that pathogenesis may occur by other means than by binding specifically to α-1,2-fucosylated receptors. Other receptors than H-2 may allow *E coli* F18 epithelial adhesion and a more efficient inhibition of *E coli* F18 adhesion and thus decreased prevalence of diarrhea may result from supplementation with multiple HMOs and antimicrobial milk factors.

The increased luminal abundance of an unclassified Enterobacteriaceae genus in the F18 pigs (compared with controls) most likely includes the inoculated *E coli* F18. The abundance of Enterobacteriaceae in the 2FL-F18 pigs was intermediate between values in the other 2 groups, but not significantly different from either of them, indicating that 2′-FL supplementation tend to reduce the abundance of *E coli* F18. The unclassified Lachnospiraceae family was relatively more abundant in 2FL-F18 pigs, supporting the increase of this group in responses to HMO supplementation, including 2′-FL, in newborn pigs (30). The control group showed the highest abundance of *Enterococcus*, despite that these were increased in abundance after 2′-FL supplementation in preterm pigs (31). From the above, it seems that inoculation with *E coli* F18 may override any potential effect of 2-FL on *Enterococcus* density.

From the present in vitro and in vivo studies we conclude that 2′-FL reduces *E coli* F18 adhesion in the intestine of newborn, cesarean-derived, formula-fed pigs. The employed dose of 2′-FL was not sufficient to prevent diarrhea when *E coli* F18 was inoculated at a dose of 7.5×10^{10} cfu/day. Tendencies to improve intestinal morphology and health in the 2FL-F18 pigs suggest only a modest beneficial effect of 2′-FL. It remains to be investigated whether an increased dosage of 2′-FL, or a longer time of exposure in a more mature intestine, more effectively prevents pathogen-induced intestinal infections in early life of pigs and infants.

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